Studies on age-related neuroanatomical and biochemical changes in the brain of goat (*Capra hircus*)

Synopsis

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INTRODUCTION

The brain is the one of the most complex and the important organ in the human body. It is made up of almost 100 billion neurons that communicate in trillions of connections called synapses. The brain is primarily composed of the cerebrum, cerebellum, and brainstem. The brain is divided into two halves i.e., the right and left hemispheres. The surface of the cerebrum is called the cerebral cortex. The cerebral cortex majorly contains cyton of neurons (grey matter), which are interconnected to other brain areas by axons (white matter). The cerebral cortex has a folded appearance in the form of gyri and sulci.

Neuron is the structural and functional unit of the nervous system. Brain development is a protracted process that begins in the gestational period with the differentiation of the neural progenitor /cells and extends at least through late adolescence, however arguably throughout the lifespan.

By the end of the embryonic period, the rudimentary structures of the brain and central nervous system are established in the major compartments of the central and peripheral nervous systems. The ensuing period of foetal development extends through the end of gestation. During this time there is rapid growth and elaboration of both cortical and subcortical structures, including the rudiments of the major fibre pathways **(Jovanov-Milosevic *et al.*, 2006**). Brain development continues for an extended period postnatally. There are phenomenal change occurring in all parts of the body, including the brain, with the age progression.

The model organism using higher mammals can provide an important platform for the gyrencephalic and goat brain is almost like humans (*Homo sapiens*), having exquisite two hemispheres and brain parts. The goat has a definitive grey to white matter ratio and a brain growth spurt in the prenatal period that is similar to human brain. The colour and texture of the goat brain are like that of the human brain. In addition, the goat brain is less expensive than a human cadaveric brain and may be used for neuromorphological studies **(Onoda *et al*., 2022).**

Aging is the progressive loss of tissue and organ function over time. The free radical theory of aging, later termed as oxidative stress theory of aging, is based on the structural damage-based hypothesis that age-associated functional losses are due to the accumulation of oxidative damage to macromolecules (lipids, DNA, and proteins) by reactive oxygen and nitrogen species **(Liguori *et al.*, 2018)**.

Certain parts of the brain shrink, especially those important to learning and other complex mental

activities. In certain brain regions, the communication between neurons may not be as effective as blood flow in the brain may decrease with aging. However, inflammation may increase when the body responds to an injury or disease **(Nikhra, 2017; Ademuyiwa, *et al.*, 2021)**.

The normal adult brain undergoes considerable morphological changes with age progression. A decrease in the weight of brain and an increase in cerebrospinal fluid (CSF) filled spaces (ventriculomegaly), caused by ventricles dilation and sulcal enlargement, have been documented by autopsy studies. The differences between white and grey matter loss in the aging brain have also been reported **(Hsu *et al.*, 2008)**. Brain aging has been linked with subtle alterations in the structure and function of neurons in specific neuronal circuitry, rather than only high rates of neuronal loss. The brain in age-associated neurodegenerative diseases, leads to loss in dendritic arbors and synaptic contacts **(Huffman, 2012)**.

Numerous pathways leading to brain aging, including increased oxidative stress, inflammation, disturbances in energy metabolism such as deregulated autophagy, mitochondrial dysfunction, IGF-1, mTOR, ROS, and p53 as central modulators of the metabolic control, lead to neurodegenerative disorders **(Zia *et al.*, 2021)**.

Acetylcholinesterase (AChE) is a large glycoprotein (about 15% carbohydrate by weight), whose basic chemistry was worked out by **Leuzinger and Baker (1967)** and others. Acetylcholinesterase hydrolyses the neurotransmitter acetylcholine at neuromuscular junctions and brain cholinergic synapses and this way terminates signal transmission. Acetylcholinesterase exists in multiple molecular forms which possess similar catalytic properties but differ in their oligomeric assembly and mode of cell attachment to the cell surface. Inhibition of acetylcholinesterase (AChE), metabolizing enzyme of acetylcholine, is presently the most important therapeutic target for development of cognitive enhancers **(Das *et al.*, 2001)**.

The catalytic serine of AChE can be phosphorylated by a soman, one of the most potent nerve agents, and subsequently undergoes an aging reaction. This phosphorylation and aging process leads to irreversible AChE inhibition, resulting accumulation of excess ACh at the synaptic cleft, and causes neuromuscular paralysis **(Sirin *et al.*, 2012)**. One of the studies had revealed a decrease in total cholinesterase in the cerebral cortex, hippocampus and striatum in aged rats (24 months) of various strains, as compared with young animals (3 months) **(Meneguz *et al.*, 1992)**.

To study these developmental changes in the brain, staining procedure are vastly used in scientific literature. It enhances visualization of the cell or certain cellular components under a microscope. Cells may be stained to highlight morphology, anatomy and metabolic processes or to differentiate between live and dead cells in a brain tissue sample.

The cytoplasm of cells is acidophilic, whereas the nuclei are basophilic in nature. Nissl bodies, which are nothing but rough endoplasmic reticulum (RER) of neurons, are basophilic, while other cytoplasmic structures are mostly acidophilic in nature.

Brain degeneration during normal aging underlies cognitive disorders experienced age progression. Not all molecular mechanisms associated with age-related neurodegeneration are fully understood. Apart from the issues raised by the attempts to expand the lifespan, understanding the mechanisms and the detailed metabolic interactions involved in the research of normal neuronal aging continues to be a challenge.

Is there neuronal loss or changes in the neuronal structures during the age progression? Do the number of neurons remain same throughout the life or these increase or decrease during the chronological age? Is there any change in the biochemical level, along with anatomical changes in the brain during aging? The proposed work is to study the age-associated neuroanatomical and biochemical changes in the goat brain during age-progression.

REVIEW OF LITERATURE

To the as early as year of **1935,** hypophysectomy of the milking goat brain was reported by the **Hill** in which the pituitary was removed from the brain of the goat and also elaborated the operative techniques and discussed the changes in lactation in goats due to removal of the pituitary gland. **Clark *et al.*** in **1941** experimented on cortical stimulation in goat brain related to myotonia and clonic movements. **Adrian** in **1943** experimented over the brain of ungulates and reported afferent signalling in the goat brain. In the goat and sheep, the chief supply to the cortex comes from the upper and lower lips on the same side. The fore-foot and sometimes the hind-foot sends discharges to an area on the caudal side of the face area in the anterior part of the ectosylvian gyrus. **Yamamoto** in **1952** studied on comparative analysis on Brainstem of animals. The pyramidal tract was found particularly poor in the transversal section of brainstem in the goat (on 4th day after birth) and similarly found a considerable difference in

the development of the pyramidal tract among other animals. **Andersson and Jewell (1956)** explored the distribution of carotid and vertebral blood supply in the brain and spinal cord of the goat. Almost the entire brain is supplied by blood from the carotid arteries, and receives no contribution from the vertebral arteries.

Bligh *et al.* (1971) experimented on influence of ambient temperature on the thermoregulatory responses to 5-hydroxytryptamine, noradrenaline and acetylcholine injected into the lateral cerebral ventricles of sheep, goats and rabbits. On goat, the effects on thermoregulation of the i.c.v. (intracerebroventricularly) administration of these substances were dose dependent. Acetylcholine in doses of upto1mg (about 28ug/kg), rarely evoked even a brief response. But if 250 ug Ach were injected 20 min later there was an increase in EMG activity at ambient temperatures of 200°C and below, and a reduction in respiratory frequency at ambient temperatures of 200° C and above.

Age-related changes in acetylcholinesterase and its molecular forms in various brain areas of rats had studied by **Meneguz *et al.* (1992)** which showed that there were two molecular forms of AChE, namely G4 (globular tetrameric) and G1 (monomeric). Since G4 forms have been proposed to be present pre-synaptically, their age-related loss in those brain areas where acetylcholine plays an important role in neurotransmission, may indicate an impairment of presynaptic mechanisms. **Lieberman *et al.* (2001)** had studied a longitudinal study of brain morphology in first episode of schizophrenia and results confirmed the findings of ventricular enlargement and anterior hippocampal volume reduction in first episode of schizophrenia patients that have been previously reported. In addition, the persistence positive and negative changes in selected structures over time, including increases in ventricular volume that were associated with poor outcome patients were possibly due to neural degeneration.

DeCarli *et al*. (2005) had worked on the measures of brain morphology and infarction in the Framingham Heart study and observed that frontal lobe volumes showed the greatest decline with age (approximately 12%), whereas smaller differences were found for the temporal lobes (approximately 9%). Age-related differences in occipital and parietal lobe were modest. Age-related gender differences were generally small, except for the frontal lobe where men had significantly smaller lobar brain volumes throughout the age range studied. **Pugliese *et al.* (2007)** had explored in a paper entitled "canine cognitive dysfunction and the cerebellum: acetylcholinesterase reduction, neuronal and glial changes"

with results showing the highest acetylcholinesterase optic density corresponded to cerebellar islands of the granular layer and was predominantly associated with synaptic glomeruli and the somata of Golgi cells. Its reduction correlated with aging and loss of granule cells, whereas cognitive deficit only correlated with loss of Purkinje cells.

Lucy *et al.* (2008) had worked on prenatal development of neurons in the brain of goat. This work was undertaken to study the origin and developmental pattern of neurons in the brain of goats during prenatal period. In **2009, Jha and Rizvi** had explored age-dependent decline in erythrocyte acetylcholinesterase activity and correlated with oxidative stress and found decreased antioxidant defence, and alteration in membrane rheology during aging process through decreased activity of AChE in erythrocyte membrane. This finding could help in explaining the neuronal complications taking place under conditions of oxidative stress, aging, and dementia.

Lemaitre *et al.* (2012) had worked on normal age-related brain morphometric changes to test nonuniformity across cortical thickness, surface area and grey matter volume and analysed through structural images of 216 healthy volunteers, ranging from 18 to 87 years of age, using a surface-based automated parcellation approach. They found that linear regressions of age revealed a concomitant global age-related reduction in cortical thickness, surface area and volume. **Schliebs and Arendt (2011)** had explored the cholinergic system in aging and neuronal degeneration and concluded that aging-related cognitive impairments are due to loss of cholinergic function. In inherited and late Alzheimer's disease, basal forebrain cholinergic cells degenerate and mild cognitive impairment characterized by loss of cholinergic function. In a research paper entitled "clinical neurogenetics: autosomal dominant spinocerebellar ataxia" had explored that overall brain shrinkage as we age and it starts in the age of 30s and 40s, but the volume loss was not uniform throughout the brain, some areas shrink more, and faster, than other areas by **Shakkottai and Fogel (2013)**. **Briefer *et al.* (2014)** studied the cognitive tasks in goats by assessing the learning and remembering capability and observed that most trained goats (9/12) successfully learned the task quickly on average, within 12 trials. After intervals of up to 10 months, they solved the task within two minutes, indicating excellent long-term memory.

Haider *et al.* (2014) had observed age-related learning and memory deficits in the rats associated with the role of altered brain neurotransmitters, acetylcholinesterase activity and changes in antioxidant

defence system and the data suggested that increased oxidative stress, decline of antioxidant enzyme activities, altered AChE activity, and decreased biogenic amines level in the brain of aged rats may potentially be involved in diminishing memory function. **Janeczek *et al*. (2018)** had explored variations in acetylcholinesterase activity within human cortical pyramidal neurons across age and cognitive trajectories and reported that low levels, if AChE activity could enhance the impact of acetylcholine on pyramidal neurons to counterbalance other involutional factors that mediate the decline of memory capacity during average aging. **Sharma *et al.* (2019)** had studied adult goat retinal neuronal culture. It resulted to be a great model for studying the neurodegenerative changes in adult retina and it is easier accessible to detect neuronal abnormalities in animals.

Current state of knowledge on the cognitive capacities of goats and its potential to inform species-specific enrichment and observed that goats have developed the cognition to cope with the harsh foraging and dynamic social conditions. They have developed the cognitive ability to make decisions **(Zobel and Nawroth, 2020**). **Raina (2021)** had researched over sleep deprivation related to brain acetylcholinesterase activity and cognitive ability in young and aged rats and observed that the most affected part of the brain was hippocampus followed by cerebral cortex. Acetylcholinesterase was found to be altered in sleep deprivation treated rats. Results were remarkably correlated with the cognitive impairment.

Wrigglesworth *et al.* (2021) had reviewed the factors associated with brain ageing and gave this systematic review to identify and synthesize the evidence for an association between lifestyle, health factors and diseases in adult populations, with brain ageing. **Zia *et al.* (2021)** had studied molecular and cellular pathways contributing to brain aging. The recent evidence about the modern molecular and cellular methods in neurobiology for brain aging is that neocortex and hippocampus are the most vulnerable areas, affected in aging with a variable degree of molecular and cellular alterations. Physical exercise and calorie restriction also exhibit a significant potential in brain cells resistance to neurodegenerative disorders.

After studying above literature survey and other associated articles, we have proposed the following objectives to study the age-related neuroanatomical and biochemical changes in the brain of goat during age progression.

OBJECTIVES OF THE STUDY

1. To study the age-related changes in size, weight, volume, and ventricle size in goat brain.
2. To study the anatomical changes in the brain (frontal lobe, hippocampus & cerebellum) with respect to aging by staining neuronal and neuroglial cells.
3. To study the age-related biochemical changes (Acetylcholinesterase activity) in goat brain.

MATERIAL AND METHODOLOGY

Material-

- **Collection of samples-**

Healthy goat brains will be collected from the nearby slaughterhouse(s) and/or from the Central Institute for Research on Goats (CIRG), Makhdum, Mathura. We have no intention to treat or give any medication to the goats and only the goat brain of various age progression shall be utilized for the study. Goat brains of various ages shall be used for the study (6 months to 12 years).

Identification marker of age by teeth **(Cooper T., 2020)**: -

1. Central pair: 1–1.5 years old
2. 2nd pair: 1.5–2 years old
3. 3rd pair: 2.5–3 years old
4. 4th pair: 3.5–4 years old
5. Signs of wear: 4+ years old
6. Spreading: 5+ years old
7. Lost or broken: 6+ years old

Table 1: - Sample size

Age (From 6 months -12 years)	**Sample size (24)**	**Blocks per sample (24x3)**
6 months	x4	x3
1 year	x4	x3
2 years	x4	x3
4 years	x4	x3
8 years	x4	x3
12 years	x4	x3

- **Fixation of tissue-**

The goat brain shall be transported to the laboratory in cold phosphate-buffered saline (137 mM NaCl,

2.7 mM KCl, 8 mM Na_2HPO4, and 2 mM KH_2PO4; pH 7.4) and/or physiological saline and shall be processed as early as possible for further experiments. The brain will be taken for lobotomy to cut into blocks of cerebrum, hippocampus and cerebellum and will be fixed in 10% neutral buffered formalin. The blocks of brain tissue will be taken for the neuroanatomical study. The fixed tissues will be embedded in paraffin wax, followed by microtomy to visualize neurons and glial cells (Reference URL of Goat brain Atlas: Sunagawa, K., Nagamine, I., Fujino, T., Nakatsu, Y., & Hikosaka, K. (2015). Stereotaxic atlas of the goat brain for an accurate approach to the hypothalamic nuclei. *Physiology & Behavior*, *145*, 91-105.).

- **Neurostaining**

a). Holzer's crystal violet stain: -

This stain shall be used for the glial cells. This method stains fibrous astrocytic processes bluish purple with crystal violet. The proliferation of astrocyte fibrous components in response to tissue damage and change in the glial cells number can be observed with this stain **(Bayram-Weston *et al*., 2016)**.

b). Cresyl violet stain: -

Cresyl violet acetate solution is used to stain Nissl's substance in the cytoplasm of neurons in paraformaldehyde or formalin-fixed tissue. The cell body of neurons shows staining a granular purple blue. This stain is one of standard methods to identify the neuronal structure in brain and spinal cord **(Zhu *et al*., 2015)**.

c). Golgi stain/ Cajal stain: -

Golgi's method is a silver staining technique that is used to visualize nervous tissue under light microscopy. This can homogeneously stain neural cell structures such as axons, cell bodies, dendrites, and dendritic spines, so that morphologic details can be clearly visualized. The foremost method was discovered by Camillo Golgi who published the first pictures made with the technique in 1873 **(Rabanal *et al*., 2015)**. Now modified methodology of rapid Golgi's staining is available for quick and efficient visualization **(Patro *et al*., 2013)**.

Methodology

- **Size/ weight ratio-** Size, weight and volume ratio will be measured to observe the differences in the brain of goat at different ages.

- Brain tissue shall be brought to the lab in cold PBS and/or physiological saline.
- Precise brain weight and parts of the brain will be measured on electronic balance.
- Physical measurement of cortical parts will be done with the help of thread and scale.
- Ventricle measurement will be done with the help of CT scan (neuroimaging facility).

➢ **Holzer's crystal violet staining method: -**

Sample will be deparaffinized and hydrated with PBS (7.4 pH, 1M). Then sample will be treated with phosphomolybdic acid-alcohol working solution. Alcohol-chloroform solution will be used to make the sample translucent. After that crystal violet solution will be used. Then potassium bromide solution will be used followed by differentiating solution. The sample will be rinsed with xylene. The neuroglia fibres will be visualized with deep violet-blue coloured, and background will be pale violet. This will demonstrate glial cells distribution in the brain tissue in age progression.

➢ **Cresyl violet staining method: -**

First sample will be rehydrated with the alcohol. The stain with the Cresyl violet stain followed with the ethanol wash. The Cresyl violet staining gives dark blue colour to Nissl's substance (rough endoplasmic reticulum). This will help in visualizing the difference in the distribution of neurons via staining Nissl's substances as age progresses.

➢ **Golgi staining (Cajal staining) method: -**

Formol fixed brain tissue will be immersed into aqueous solution of potassium dichromate. After drying sample will be immersed into aqueous solution of silver nitrate then section will be cut and will be dehydrate with ethanol, clear and mounted.

This technique has since been refined to substitute the silver precipitate with gold by immersing the sample in gold chloride then oxalic acid, followed by removal of the silver by sodium thiosulphate. This preserves a greater degree of fine structure with the ultrastructural details marked by small particles of gold. The Golgi stain or Cajal stain gives black colour to neuronal circuitry in the brain. Golgi staining will help in the observation of the neuronal structure in the brain with age progression.

➢ **Microtomy: -**

Goat brain shall be obtained from the slaughterhouse and through the standard process of dehydration and paraffin block preparation shall be performed. Microtomy of sections shall be done in the

microtome available at the department over the slides and reserved for further processing. For the complete neuron staining, similarly semi-thin sections of brain tissue shall be prepared.

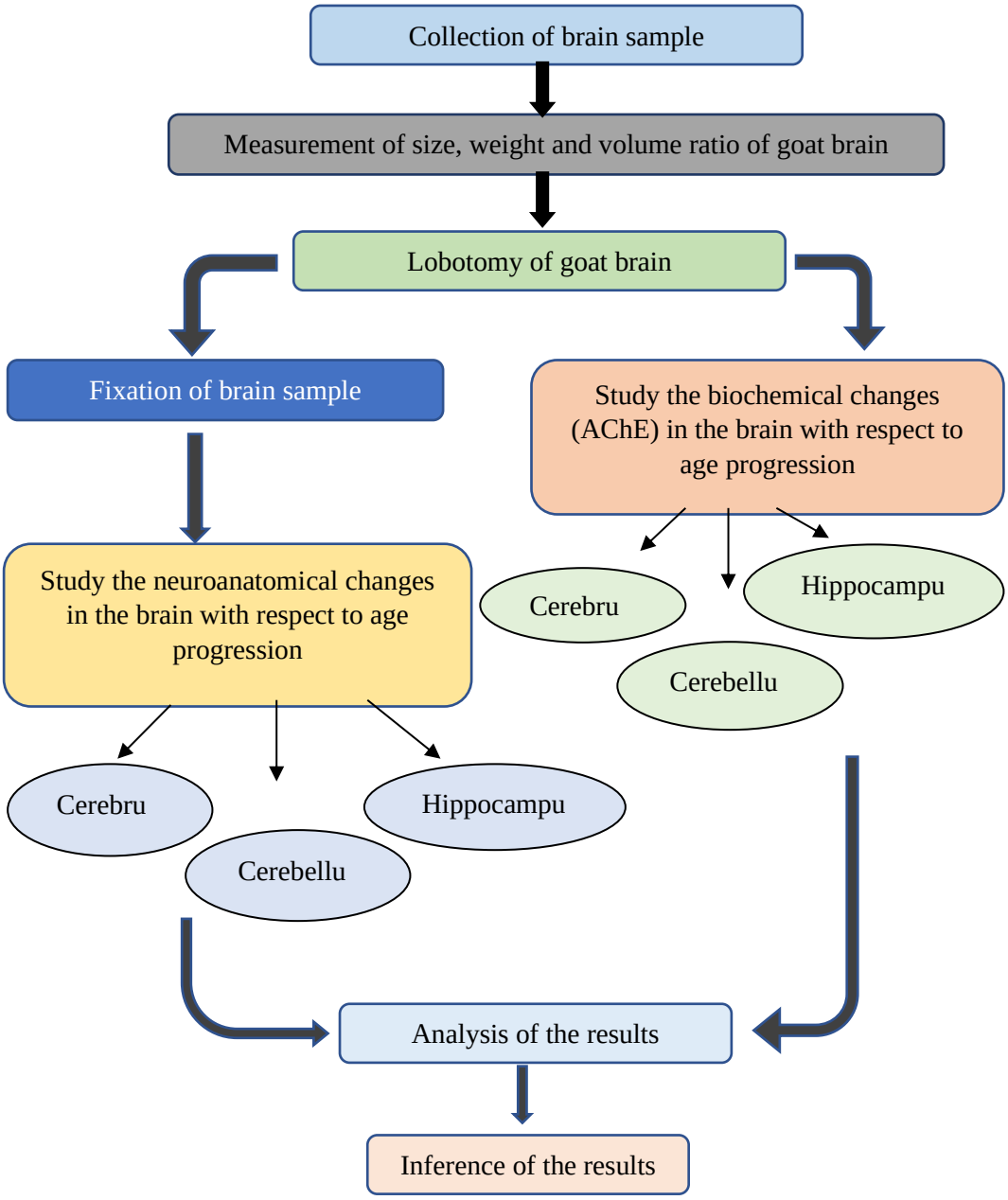
- **Acetylcholinesterase activity: -**

Acetylcholinesterase activity will be assayed spectrophotometrically in the different brain regions of the goat of different ages.

Activity of acetyl cholinesterase (AchE) in brain and plasma will be determined according to the method of **Ellman *et al*. (1961)** using acetylthiocholine (ATC) as substrate. The reaction mixture contained brain homogenate, phosphate buffer, and DTNB. The reaction mixture will be mixed by bubbling air and placing in the spectrophotometer. Once the reaction is stable, the absorbance will be recorded at 412nm for the basal reading. The reaction will be started by adding ATC to this cuvette, and change in absorbance will be recorded at time zero and after 10 minutes at 25°C. The activity of acetylcholinesterase will be expressed for as micromoles per minute per gram of brain or micromoles per minute per milliliter of plasma **(Haider *et al*., 2014)**.

- **Statistical analysis-**

Mean value and standard error will be calculated in the selected region of the brain tissue of goat of different age groups. The significance of difference between the values of groups will be determined by ANOVA test.


Collection of brain sample
Measurement of size, weight and volume ratio of goat brain
Lobotomy of goat brain
Fixation of brain sample
Study the biochemical changes (AChE) in the brain with respect to age progression
Study the neuroanatomical changes in the brain with respect to age progression
Cerebru
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Cerebru
Hippocampu
Cerebellu
Analysis of the results
Inference of the results

Fig-1: - FLOW CHART

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